Aurora Kinase-A Deficiency during Skin Development Impairs Cell Division and Stratification

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Aurora kinase-A (Aurora-A) promotes timely entry into mitosis, centrosome maturation, and formation of bipolar spindles. To address the role of Aurora-A in skin development and homeostasis, we interbred a floxed Aurora-A (*Aurora-A*^{fl}) mouse with the Cre-deleter strain, *K14.Cre*. *Aurora-A*$^{fl/fl}$;*Krt14.Cre* (*Aurora-A*$^{-/-}$) mice died shortly after birth. These mice had translucent skin, and histological evaluation showed that the dorsal skin was very thin and fragile with frank erosions. Although the expression of the basal layer marker keratin 14 and the differentiation marker keratin 1 was evident in *Aurora-A*$^{-/-}$ epidermis, there was a marked reduction in the number of suprabasal layers and basal keratinocytes. Dye exclusion assays also showed defects in barrier function. Unlike wild-type cells, *Aurora-A*$^{-/-}$ basal progenitors were delayed in forming two layers at embryonic day (E)13.5 when embryonic skin begins to stratify. Increased numbers of mitotic cells, apoptotic bodies, and polyploid keratinocytes were evident in *Aurora-A*$^{-/-}$ epidermis, indicating that a deficiency in Aurora-A promotes aberrant mitosis, mitotic slippage, and cell death. Finally, *Aurora-A*$^{-/-}$ keratinocytes displayed centrosomal abnormalities that included centrosomes located at nonapical sites in basal cells. Thus, the deletion of *Aurora-A* in the developing epidermis alters centrosome function of basal keratinocytes and markedly impairs their ability to divide and stratify.

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INTRODUCTION

Aurora kinase-A (Aurora-A) belongs to a family of conserved mitotic serine/threonine kinases that have fundamental roles in the regulation of cellular division. Aurora-A is found predominantly at the poles of dividing cells, localized to the centrosomes and proximal spindles. It is required for the timely entry into mitosis, centrosome maturation, formation of bipolar spindles, and has been implicated in the breakdown of the nuclear envelope and the completion of cytokinesis (Marumoto *et al.*, 2003; Vader and Lens, 2008). Moreover, a role for Aurora-A was recently demonstrated in the control of mitochondrial fission during mitosis (Kashatus *et al.*, 2011). Its protein stability and activity are tightly coupled to the stages of the cell cycle, peaking at the G2/M transition and suppressed at anaphase through protein degradation that is mediated by the Anaphase-promoting complex (Honda *et al.*, 2000; Littlepage and Ruderman, 2002). Numerous interacting factors such as AJUBA, BORA, NEDD9, PAK, PLK1, and TPX2 have been identified as important for Aurora-A's activation and cellular localization (Karthigeyan *et al.*, 2011). Downstream targets of Aurora-A are diverse and growing (Kettenbach *et al.*, 2011; Koch *et al.*, 2011). Of these, Aurora-A can phosphorylate the cell cycle regulator CDC25B and thereby affect the centrosomal redistribution of CDK1–Cyclin B1 complexes to promote mitotic entry (Dutertre *et al.*, 2004). Aurora-A can also activate the tubulin deacetylase, HDAC6, to regulate cilia disassembly (Pugacheva *et al.*, 2007), and phosphorylate NDEL1 to promote neurite extension (Mori *et al.*, 2009). Thus, Aurora-A has cellular functions that go beyond its ability to regulate cell division.

Aurora-A is also a proto-oncogene that is frequently amplified or overexpressed in epithelial cancers including skin squamous cell carcinomas (SCCs) (Clausen *et al.*, 2006; Torchia *et al.*, 2009). Aurora-A was further identified as a low-penetrance cancer susceptibility gene in both humans and mice (Ewart-Toland *et al.*, 2003, 2005). Ectopic Aurora-A expression can transform immortalized rodent cell lines *in vitro* and promote their growth in immunocompromised mice (Bischoff *et al.*, 1998; Zhou *et al.*, 1998). We have previously shown in genetically engineered cancer mouse models that Aurora-A can cooperate with a classical skin chemical carcinogenesis protocol or with the expression of a gain-of-function $p53^{R172H}$ mutant allele to promote the formation of metastasis-prone skin SCCs (Torchia *et al.*, 2009, 2012). Owing to its role in cell division, Aurora-A may be an attractive target for therapeutic intervention in skin cancers. Currently, there are several small-molecule inhibitors to Aurora kinase-A and -B in clinical trials (Green *et al.*, 2011). However, the long-term off-tumor effects of these inhibitors remain undefined, especially in

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Abbreviations: Aurora-A, Aurora kinase-A; BrdU, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; SCCs, squamous cell carcinomas; Tam, tamoxifen; WT, wild type

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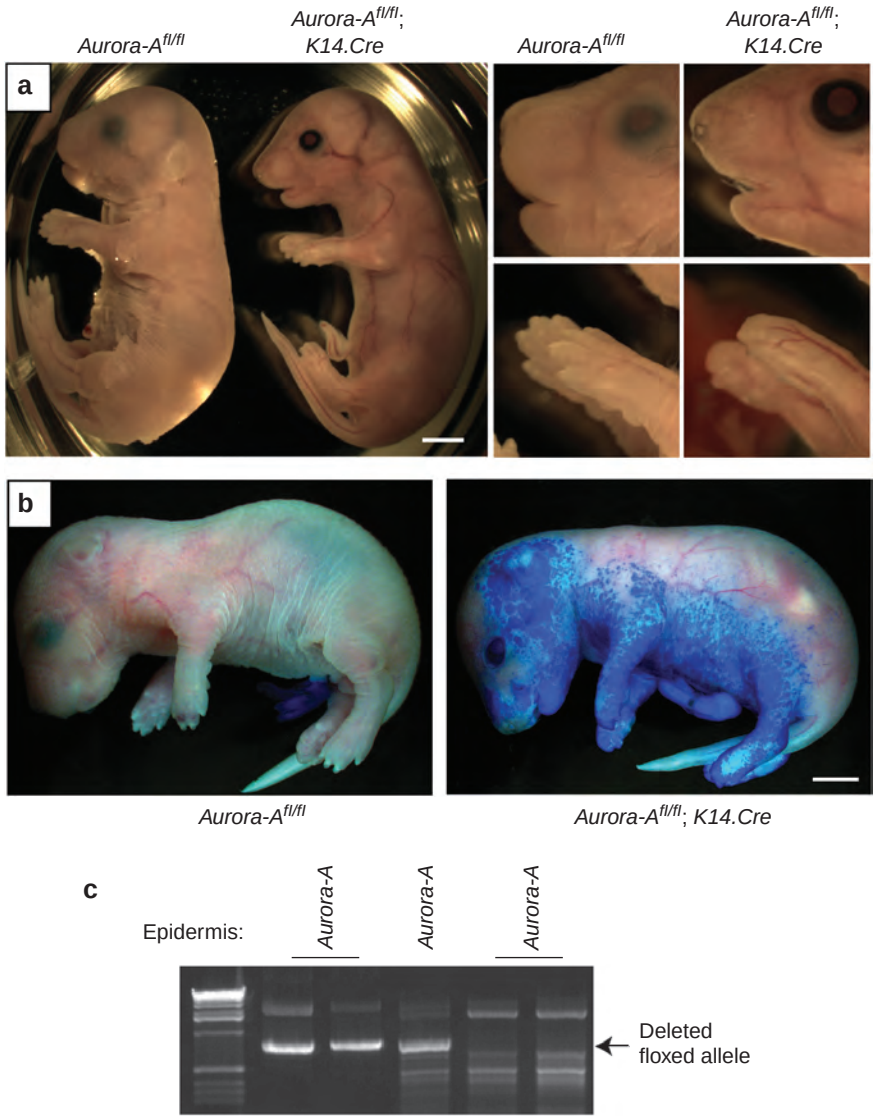


Figure 1. Skin abnormalities in mice deficient for epithelial Aurora-A. (**a**) Left panels show gross phenotypic presentation of *Aurora-A*$^{fl/fl}$; *K14.Cre* mice at embryonic day (E)18.5. These mice show translucent skin compared with wild-type (WT) controls. Right panels show a close-up of the face and paws. Note the improperly formed whiskers, nares, eyelids, and nails. (**b**) Toluidine blue dye penetration assays revealed a defect in barrier function in *Aurora-A*$^{fl/fl}$; *K14.Cre* E18.5 mice. (**c**) Tails from newborn mice were digested and PCR performed to detect Cre-mediated recombination of the floxed Aurora-A allele. Bars = 2.5 mm.

skin. Inhibition of Aurora-A activity in cultured cells results in mitotic abnormalities, misalignment of chromosomes, and growth suppression (Barr and Gergely, 2007). Recently, three different laboratories have described the global deletion of *Aurora-A* in mice (Lu *et al.*, 2008; Sasai *et al.*, 2008; Cowley *et al.*, 2009). In these reports, deletion of *Aurora-A* resulted in embryonic death before implantation (Lu *et al.*, 2008; Sasai *et al.*, 2008; Cowley *et al.*, 2009). From these studies, it is clear that Aurora-A is essential for life, but the early lethality of these mice precluded the study of Aurora-A's function in tissue-specific development or homeostasis. In the present study, we examined the consequences of keratinocyte-specific deletion of *Aurora-A* to determine its role in the formation and maintenance of skin epithelia.

RESULTS

Skin-specific deletion of *Aurora-A*

To delete Aurora-A in skin, we interbred floxed *Aurora-A* mice (Sasai *et al.*, 2008) with a constitutively active Cre recombinase expressed from a truncated keratin 14 promoter (K14.Cre) (Dassule *et al.*, 2000). This Cre deleter strain begins to express Cre in epithelial tissues at embryonic day (E)10.5 and displays Cre activity more uniformly by E12.5 as shown by the activation of the Rosa26 β-galactosidase Cre reporter (Supplementary Figure S1a online). *Aurora-A*$^{fl/+}$; *K14.Cre* mice appeared phenotypically normal. In contrast, *Aurora-A*$^{fl/fl}$; *K14.Cre* mice died shortly after birth, and had obvious skin-related abnormalities including fragile, translucent, and stretched skin, the failure of eyelids and ears to form normally, fused fingers, and the absence of whisker follicles

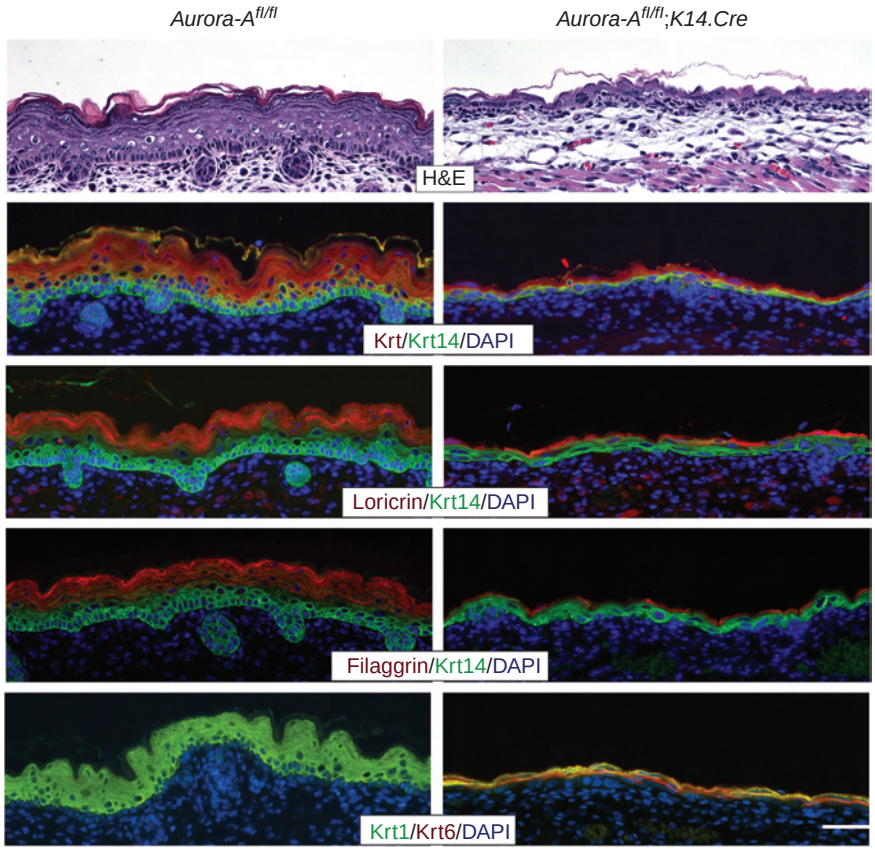


Figure 2. *Aurora-A$^{fl/fl}$;Krt14.Cre* (*Aurora-A$^{-/-}$*) epidermis exhibits a disrupted epidermal architecture. Histological analysis (hematoxylin and eosin (H&E)) of *Aurora-A$^{-/-}$* dorsal skin at embryonic day 16.5 revealed a thin epidermis and the absence of mature hair follicles. However, marker analysis showed the presence of a basal layer by the detection of keratin 14 (Krt14) expression, whereas expression of Krt1 marked the presence of the suprabasal compartment. A similar pattern was observed for Krt10/Krt 5 expression (not shown). The late differentiation markers, loricrin and filaggrin, were visible in the outermost part of *Aurora-A$^{-/-}$* epidermis. DAPI, 4′,6-diamidino-2-phenylindole. Bar = 25 μm.

(Figure 1a). Moreover, toluidine penetration assays in E18.5 embryos revealed defects in skin barrier function, which were more pronounced on the ventral side of the embryos (Figure 1b). Detection of the recombined floxed allele was evident in the skin of both *Aurora-A$^{fl/+}$; K14.Cre* and *Aurora-A$^{fl/fl}$; K14.Cre* newborn mice (Figure 1c and Supplementary Figure S1b online). Deletion of the floxed allele was associated with reduced Aurora-A mRNA levels in whole skin (Supplementary Figure S2a online) and the absence of detectable Aurora-A protein in actively dividing keratinocytes of *Aurora-A$^{fl/fl}$; K14.Cre* embryos (Supplementary Figure S2b online). Collectively, these observations indicate that a defective skin barrier, caused by epidermal Aurora-A deficiency, most likely led to desiccation and perinatal lethality of *Aurora-A$^{fl/fl}$; K14.Cre* newborn mice.

Histological evaluation of the skin of *Aurora-A$^{fl/fl}$; K14.Cre* mice at E16.5 and older showed a thin epidermis and the absence of mature hair follicles (Figure 2). We focused on the interfollicular keratinocyte compartment for the remainder of this study. The nuclei of the *Aurora-A$^{-/-}$* keratinocytes appeared enlarged with multiple nucleoli. Marker analysis revealed the expression of keratin (Krt)1 and 10 in the suprabasal compartment, whereas Krt14 and Krt5, and the stress keratin Krt6, were evident in the basal layer of *Aurora-A$^{fl/fl}$; K14.Cre* dorsal skin (Figure 2). The late differentiation markers, loricrin and filaggrin, were also detectable in the outermost layer of *Aurora-A$^{-/-}$* epidermis. However, the expression of filaggrin was markedly reduced or sporadic (Figure 2). A similar pattern of expression was observed for Krt1 on the ventral side of E17.5 *Aurora-A$^{fl/fl}$; K14.Cre* embryos, whereas loricrin and filaggrin expression was markedly reduced or absent (Supplementary Figure S3 online). The lack of loricrin and filaggrin expression on the ventral side likely contributed to a more severe impairment of barrier function in *Aurora-A$^{fl/fl}$; K14.Cre* embryos (see Figure 1b). Both collagen IV and integrinα6 were found at the epidermal–dermal junction of Aurora-A-deficient epidermis, and we observed an expression pattern similar to wild-type (WT) for the adherent junction proteins Zo-1, desmoglein 1/2, and desmocolin3 (data not shown). Thus, Aurora-A deficiency did not disrupt the integrity of the basement membrane, or cellular junctions of the epidermis.

Delayed stratification of *Aurora-A$^{-/-}$* skin epithelia

The phenotypic presentation and abnormal epidermal architecture of *Aurora-A$^{fl/fl}$; K14.Cre* prenatal mice suggested

that Aurora-A deficiency may impair the formation of the stratified layers of the skin. We therefore examined the embryonic stages between E12 and 14, when the epithelium stratifies to create the suprabasal compartment (Koster and Roop, 2007). Unlike WT cells, *Aurora-A*$^{-/-}$ epidermal progenitors were delayed in forming a suprabasal layer in both the ventral and dorsal skin, as evident by histological evaluation and the late appearance of Krt1-expressing cells (Figure 3a). Because suppression of proliferation could account for a delay in the emergence of suprabasal keratinocytes, we examined the S and M phases of the cell cycle in developing skin. At E13.5, similar numbers of keratinocytes in both WT and *Aurora-A*$^{-/-}$ epidermis were positive for bromodeoxyuridine (BrdU) incorporation, indicating that Aurora-A deficiency did not alter the ability of keratinocytes to enter S phase (Figure 3c). Next, we stained for phospho-histone H3 to determine the relative number of cells in mitosis (Tapia *et al.*, 2006). As shown in Figure 3b and c for WT epidermis, only a few basal and suprabasal layer cells were positive for phospho-histone H3, consistent with a short duration spent tranversing M phase (Dover and Potten, 1988). In contrast, a much higher number of positive cells were found in *Aurora-A*$^{-/-}$ versus WT skin (a 3- and 6-fold increase in the dorsal and ventral epidermis, respectively). Furthermore, mitotic cells in *Aurora-A*$^{-/-}$ epidermis lacked a fully formed nuclear envelope as shown by a diffused staining pattern for Lamin A/C, a nuclear membrane structural component (Margalit *et al.*, 2005) (Supplementary Figure S4 online). As the nuclear envelope is disassembled before metaphase in mitosis (Margalit *et al.*, 2005), Aurora-A-deficient keratinocytes appear to stall at prometaphase of the cell cycle.

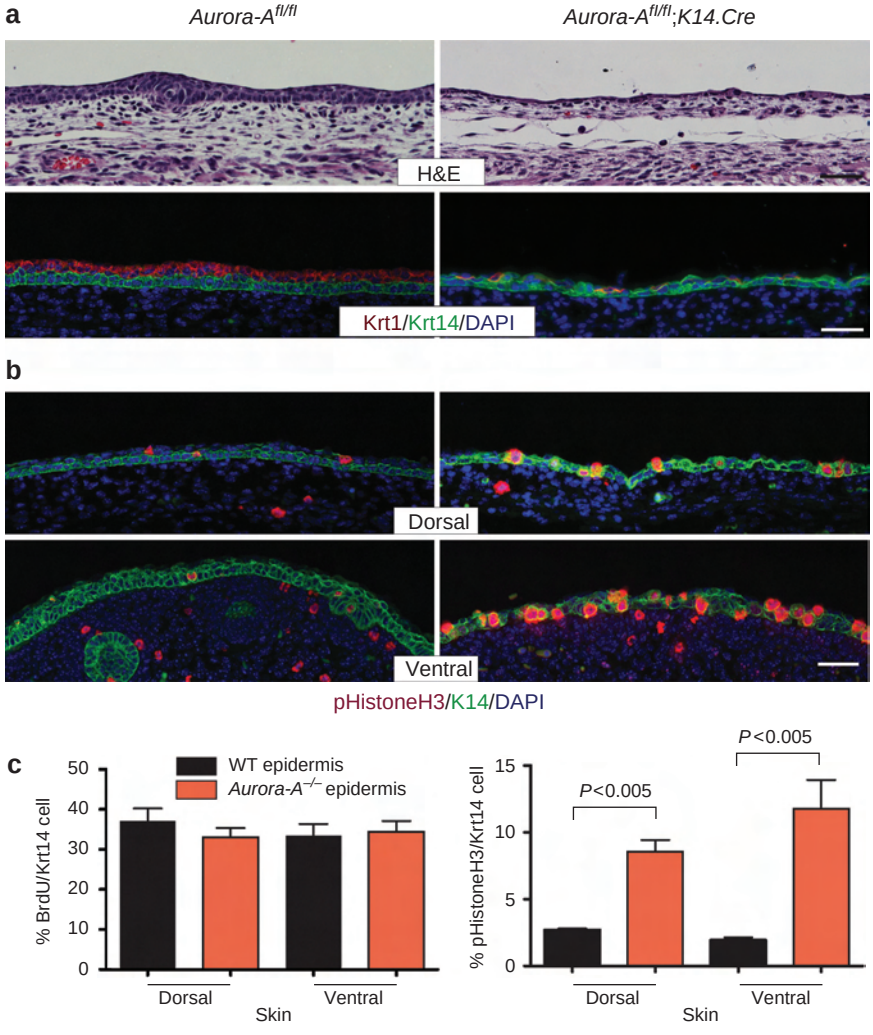


Figure 3. Delayed stratification in *Aurora-A*$^{fl/fl}$*;Krt14.Cre* (*Aurora-A*$^{-/-}$) epidermis. (**a**) The epidermis between E13 and 14 was examined by histology and marker analysis. Unlike wild-type (WT) epidermis that forms two layers at E13.5, *Aurora-A*$^{-/-}$ epidermis remains primarily a single layer. Consistently, few cells expressed the suprabasal marker keratin 1 (Krt1) in the uppermost layer of the skin. A similar pattern was observed on ventral skin (not shown). (**b**) Immunofluorescent detection of phopho-histone H3 (pHistoneH3; Ser10) in E13.5 skin. Note the increased number of positive cells found in both dorsal and ventral skin of *Aurora-A*$^{fl/fl}$; *K14.Cre* mice. (**c**) Quantification of BrdU incorporation and detection of mitotic cells per Krt14-positive keratinocytes. Data shown are the average counts per three embryos ± SD. *P*-values are from a *t*-test. DAPI, 4′,6-diamidino-2-phenylindole; H&E, hematoxylin and eosin. Bar = 25 μm.

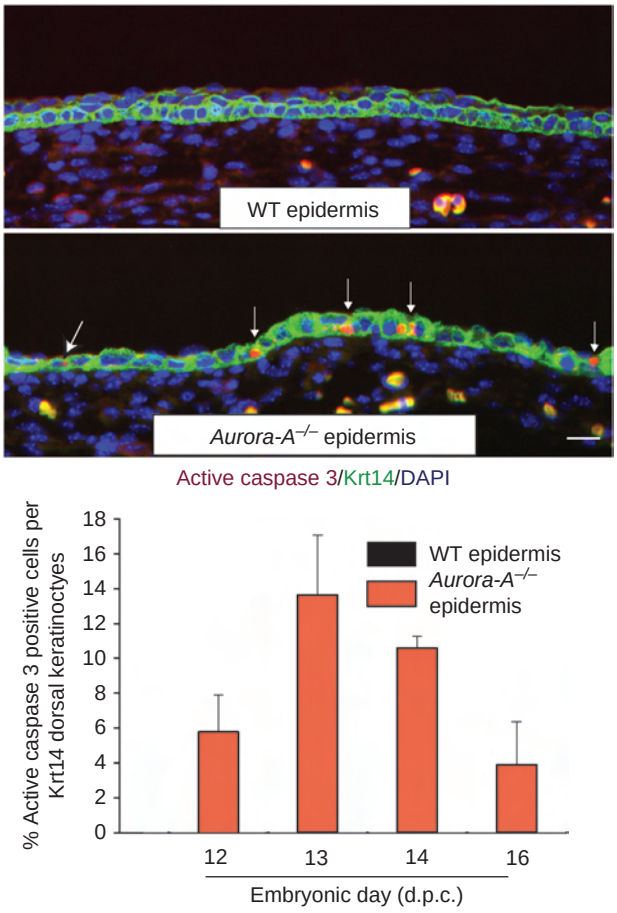


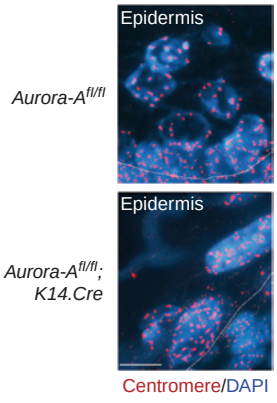
Figure 4. Increased apoptosis in Aurora-A-deficient epidermis. Top panel shows the immunodetection of active caspase 3 in wild-type (WT) and *Aurora-A$^{fl/fl}$;Krt14.Cre (Aurora-A$^{-/-}$)* epidermis at E13.5. Bottom panel shows the quantification of apoptosis found in developing skin of *Aurora-A$^{fl/fl}$; K14.Cre* or WT embryos ($n=2$ embryos per genotype and time point). Columns represent average ± SD. Note the absence of apoptotic cells in WT skin at any embryonic stage. DAPI, 4′,6-diamidino-2-phenylindole; d.c.p., days post coitum. Bar = 25 μm.

The outcome of a stalled mitosis in *Aurora-A$^{-/-}$* keratinocytes

Cells that are stalled in mitosis can undergo cell death or escape without completion of cytokinesis by a process called mitotic slippage (Rieder and Maiato, 2004). To determine whether apoptosis was a consequence of deleting Aurora-A in keratinocytes, we stained *Aurora-A$^{-/-}$* epidermis for the apoptotic marker active caspase 3. Unlike WT skin, active caspase 3 was readily detectable in *Aurora-A$^{-/-}$* epidermis. Its detection peaked at E13.5, which coincided with the commencement of stratification (Figure 4). Another outcome of a delayed mitosis is the failure to generate two daughter cells, leaving the cell with twice the normal complement of genetic material. To test this possibility, we enumerated centromeres by staining paraffin sections of embryonic skin with a pan-centromere fluorescence *in situ* hybridization probe. Increased numbers of centromeres were found in *Aurora-A$^{-/-}$* epidermis relative to the nuclei found in WT epidermis or in the dermis of *Aurora-A$^{fl/fl}$; K14.Cre* mice that remained WT for Aurora-A (Figure 5). Moreover, cells in metaphase or anaphase were rarely observed by histology or by staining with Aurora-B, which localizes to the midzone or anaphase bridges during mitosis (Ruchaud *et al.*, 2007) (data not shown). From these analyses, we conclude that *Aurora-A$^{-/-}$* keratinocytes gave rise to polyploid cells.

Centrosome abnormalities in *Aurora-A$^{-/-}$* keratinocytes

Aurora-A promotes the maturation of centrosomes and formation of bipolar spindles in preparation for cell division (Vader and Lens, 2008). Therefore, we examined how Aurora-A deficiency affected centrosome function in the developing epidermis. In WT keratinocytes at E16.5 and older, the centrosomes were predominately found apically (Figure 6a, arrows). This orientation was altered in *Aurora-A$^{-/-}$* epidermis, with the position of the centrosomes becoming random and located either apically, basolaterally, or centrally (Figure 6a, arrows, and see Supplementary Figure S5 online). As shown in Figure 6b, there were multipolar structures or



	Epidermis		Dermis		Stage
	Mean	SD	Mean	SD	
Aurora-A$^{fl/fl}$	15.4	3.7			
Aurora-A$^{fl/fl}$;K14.Cre	42.9	10.4	13.6	2.8	E16.5
	44.3	11.2	14.3	3.5	
Aurora-A$^{fl/fl}$	17.3	3.8			
Aurora-A$^{fl/fl}$;K14.Cre	67.4	9.1	15.8	3.6	E18.5
	40.0	9.6	16.8	4.2	
Aurora-A$^{fl/fl}$	17.0	4.8			
Aurora-A$^{fl/fl}$;K14.Cre	43.5	9.6	15.6	3.9	E18.5
	37.2	8.6	14.7	4.1	

Figure 5. Aurora-A deficiency induces polyploidy in keratinocytes. Left panels show the representative fluorescence *in situ* hybridization detection of a pan mouse centromere probe on 5-μm-thick paraffin sections from E16.5 skin of *Aurora$^{fl/fl}$* and *Aurora-A$^{fl/fl}$; K14.Cre* embryos. Right, quantification of centromeres. Mean count ± SD is shown. Note the consistent number of centromeres in cells found in wild-type Aurora-A epidermis or the dermis of *Aurora-A$^{fl/fl}$; K14.Cre* mice that do not undergo Cre recombination. However, much higher numbers are detected in *Aurora-A$^{fl/fl}$;Krt14.Cre (Aurora-A$^{-/-}$)* epidermis, indicative of polyploidy. DAPI, 4′,6-diamidino-2-phenylindole. Bar = 10 μm.

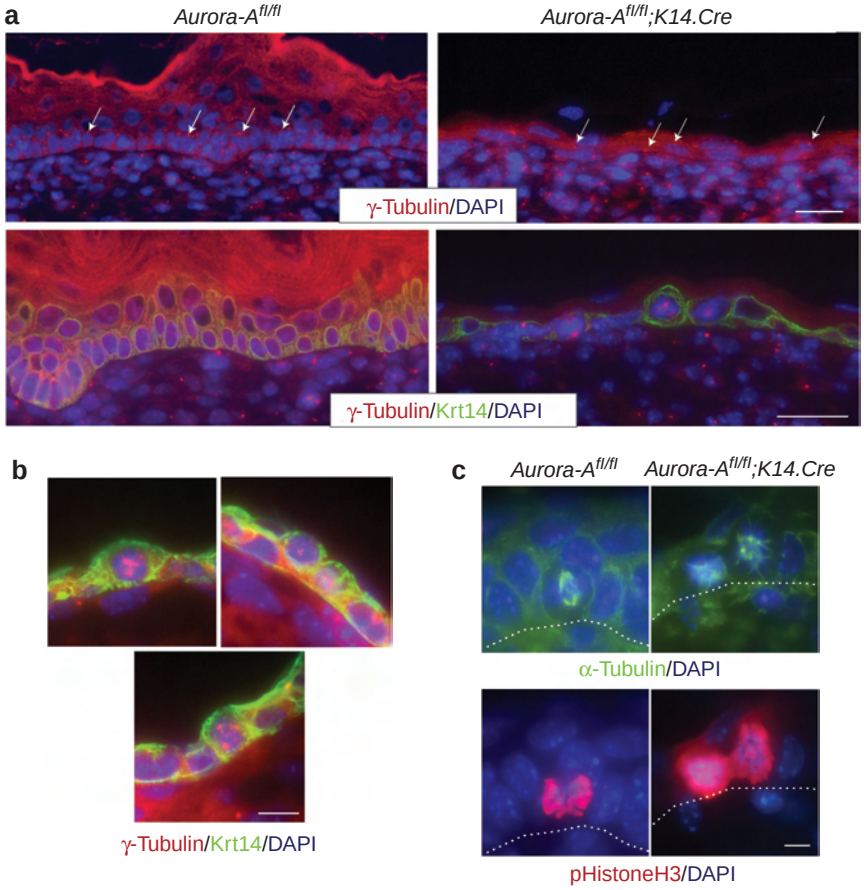


Figure 6. Centrosomal abnormalities in *Aurora-A$^{fl/fl}$;Krt14.Cre (Aurora-A$^{-/-}$)* epidermis. (**a**) The location of centrosomes was examined in *Aurora-A$^{-/-}$* E16.5 epidermal skin by immunostaining with γ-tubulin. Left panels show the apical location of centrosomes (arrows) in basal keratinocytes, relative to the basement membrane. Right panels show nonapical locations of centrosomes in *Aurora-A$^{-/-}$* epidermis (arrows). Bar = 25 μm. (**b**) Higher magnification of *Aurora-A$^{-/-}$* cells. Bar = 10 μm. (**c**) Detection of the microtubule network by α-tubulin immunostaining. Left panel shows formation of a bipolar spindle in a WT keratinocyte undergoing mitosis. Right panels show atypical microtubule formation in *Aurora-A$^{-/-}$* basal cells, reminiscent of monopolar spindles. Bar = 5 μm. DAPI,4′,6-diamidino-2-phenylindole; pHistoneH3, phopho-histone H3.

centrosome clusters in *Aurora-A$^{-/-}$* epidermis, reminiscent of spindle anomalies observed in cells with extra centrosomes (Ganem *et al.*, 2009) or those treated with Aurora-A inhibitors, and in *Drosophila* mutants of Aurora-A (Glover *et al.*, 1995; Manfredi *et al.*, 2007). Since the proper orientation of centrosomes is required for the formation of bipolar spindles in preparation for metaphase, we next examined the microtubule network in *Aurora-A$^{-/-}$* epidermis. In actively dividing WT cells, microtubules are organized between two opposing nucleating poles (i.e., the centrosomes) whose orientation to the basement membrane determines the plane of cell division (Glotzer, 2009). A basal cell that forms a bipolar spindle perpendicular to the basement membrane will divide asymmetrically and give rise to two cells, a suprabasal and a basal keratinocyte (Figure 6c) (Smart, 1970; Lechler and Fuchs, 2005). In *Aurora-A$^{-/-}$* keratinocytes, basal cells with bipolar spindles were rarely observed at E13.5 or at later embryonic stages. In dividing cells identified by phospho-histone H3 expression, the microtubule network appeared disorganized (Figure 6c). Taken together, these data indicate that abnormal centrosome function induced by the deletion of *Aurora-A* leads to a defective cellular division and the mislocation of centrosomes following mitosis of basal cells.

To determine whether the effects of Aurora-A deficiency were similar in postnatal skin, Aurora-A-floxed mice were crossed with the tamoxifen (Tam)-inducible Cre deleter strain, K14.CreER (Vasioukhin *et al.*, 1999). This Cre fusion shows robust deletion of floxed alleles in keratinocytes both *in vitro* and *in vivo* after treatment with Tam (Supplementary Figure S6a online). Similar to *Aurora-A$^{fl/fl}$; K14.Cre* embryos, Aurora-A was not detectable in dividing keratinocytes present in the skin of Tam-treated *Aurora-A$^{fl/fl}$; K14.CreER* mice (Supplementary Figure S6b online). Surprisingly, the deletion of *Aurora-A* in postnatal epidermis resulted in no obvious gross skin abnormalities (e.g., hair loss or skin erosions) 3 months following the induction of Cre activity by Tam treatment ($n = 6$ and data not shown). We were able to detect cells that were positive for phospho-histone H3 or active caspase 3 in *Aurora$^{fl/fl}$; K14.CreER* epidermis 10 days after the

induction of Cre (Supplementary Figure S7 online); however, adult epidermis appears to be reasonably tolerant to the deletion of Aurora-A.

DISCUSSION

In the present study, we utilized a conditional deletion strategy to demonstrate that the expression of Aurora-A is required for normal skin development. The deletion of *Aurora-A* in embryonic basal keratinocytes resulted in defective centrosome maturation, which markedly impaired the ability of these cells to divide and stratify. Consequently, the initiation of stratification and differentiation was delayed in the developing *Aurora-A*$^{-/-}$ epidermis, resulting in impaired barrier function and perinatal death. Previous studies have shown that barrier formation begins on the dorsal epidermis and progresses laterally starting at E16 (Hardman *et al.*, 1998). The ventral epidermis is last to acquire a competent barrier. The incorporation and cross-linking of epidermal differentiation proteins such as loricrin into the cornified envelope coincides with the formation of barrier function (Simpson *et al.*, 2011). In Aurora-A-deficient epidermis, barrier formation is defective, leading to a phenotype that is especially prevalent on ventral skin. Nevertheless, dorsal skin in *Aurora-A*$^{fl/fl}$; *K14.Cre* embryos was able to express terminal differentiation proteins and form a partial barrier, whereas the ventral epidermis was not. How dorsal skin is able to form two layers and undergo differentiation in light of an obvious cell cycle defect in basal keratinocytes is unclear. It is possible that incomplete Cre recombination of both floxed Aurora-A alleles between E12.5 and 13.5 or residual Aurora-A mRNA allowed enough basal progenitors to undergo at least one round of cellular division. Alternatively, basal delamination of nonmitotic cells may contribute to the formation of the first suprabasal layer in *Aurora-A*$^{fl/fl}$; *K14.Cre* skin (Magee *et al.*, 1987; Adams and Watt, 1990; Poumay *et al.*, 1994; Liebig *et al.*, 2009; Connelly *et al.*, 2010).

The phenotypic consequence of deleting *Aurora-A* in mouse keratinocytes is reminiscent of the effects of expression of Aurora-A mutants in *Drosophila* (Glover *et al.*, 1995; Berdnik and Knoblich, 2002). *Drosophila* mutations in Aurora-A result in lower expression of the mutant protein compared with WT flies. The most penetrant of these mutants induced monopolar spindles in dividing cells, delays in mitosis, and polyploidy (Glover *et al.*, 1995; Berdnik and Knoblich, 2002). Invariably, the failure of centrosomes to separate before metaphase can explain the effects of suppressing Aurora-A levels in flies. The deletion of *Aurora-A* in the developing mouse skin mirrored the most severe of the *Drosophila* mutants, resulting in centrosomal-related defects. In addition, the centrosomes in *Aurora-A*$^{-/-}$ basal cells appeared to lose the predominant apical placement seen in WT keratinocytes (Lechler and Fuchs, 2007) and were found to be distributed more randomly. The position of centrosomes, which determines the axis of cell division during mitosis, is set during metaphase in basal keratinocytes (Adams, 1996; Poulson and Lechler, 2010). In the stages that precede metaphase, centrosomes can be found at angles between parallel and perpendicular (0–90°) relative to the basement membrane (Poulson and Lechler, 2010). The observed locations of the centrosomes in *Aurora-A*$^{-/-}$ keratinocytes may be a consequence of these cells stalling in prometaphase, undergoing mitotic slippage, and possibly reentering the cell cycle.

Surprisingly, the postnatal deletion of *Aurora-A* resulted in no gross skin defects, suggesting that the inhibition of Aurora-A activity may be well tolerated by adult skin, and represents a clear distinction to the effects observed on the prenatal epidermis as we show in this study, or that seen in early-stage embryos (Lu *et al.*, 2008; Sasai *et al.*, 2008; Cowley *et al.*, 2009). Our observations are also consistent with recent clinical trials of Aurora-A small-molecule inhibitors that have shown increased phosho-histone H3 staining in the skin, but the absence of reported skin-related toxicities (Chakravarty *et al.*, 2011). The mechanism that allows adult skin to tolerate *Aurora-A* deletion remains unclear but may depend on the relative higher rate of proliferation observed in embryonic versus adult skin. In this regard, Aurora-A inhibition would be expected to have a more profound effect on skin cancer cells, which have a higher rate of proliferation (Torchia *et al.*, 2009), and be more similar to what we observed in embryonic keratinocytes. More importantly, our data suggest that the use of small-molecule inhibitors of Aurora-A may be a safe and viable strategy to treat recurrent or metastasis-prone skin SCCs (Ganem *et al.*, 2009) if topical delivery of inhibitors can be achieved.

In summary, we have characterized the cellular and tissue-specific changes associated with Aurora-A deficiency in the developing epidermis. Aurora-A-deficient epidermis may be an ideal model to explore the relationship between the regulation of cell division, migration, and differentiation in epidermal homeostasis, and a tool to develop new approaches to maximize the killing of SCC cells by targeting mitotic regulators.

MATERIALS AND METHODS

Generation of Aurora-A-deficient epidermis

The previously described floxed Aurora-A(fl) mice (Sasai *et al.*, 2008), K14.Cre mice (Dassule *et al.*, 2000) or K14.CreER mice (Vasioukhin *et al.*, 1999) were interbred to generate Cre-positive and heterozygous or homozygous A*urora-A*^{fl} mice. Timed matings were set up at night and the presence of vaginal plugs on the following morning noted as day 0. Embryos were then collected at days corresponding to different mouse developmental stages. The University of Colorado Institutional Animal Care and Use Committee approved animal experiments. Detection of the Cre-recombined floxed Aurora-A allele was performed as previously described (Sasai *et al.*, 2008). For experiments using the K14.CreER strain, mice were topically treated with 125 μg of 4 hydroxytamoxifen (Sigma, St Louis, MO) dissolved in ethanol or injected intraperitoneally for 6 days with tamoxifen (Sigma) (6 mg per 40 g body weight) dissolved in sesame oil.

Dye penetration and quantitative PCR assay

Dye penetration was tested on E18.5 embryos as previously described (Hardman *et al.*, 1998). Briefly, embryos were dehydrated

and rehydrated through a series of methanol washes and subsequently incubated in phosphate-buffered saline solution saturated with toluidine blue (Sigma). RNA isolation, reverse transcription, and quantitative PCR analysis was performed as previously described (Torchia *et al.*, 2009). *Aurora-A* mRNA was detected by Taqman assay# Mn01248177_m1 (Life Technologies, Grand Island, NY) and normalized to *Gapdh* (Life Technologies) levels.

Histology, immunostaining, and β-galactosidase staining

Mouse tissues were fixed in 10% neutral-buffered formalin (Thermo Fisher Scientific, Pittsburgh, PA), and embedded in Optimal Cutting Temperature compound (Sakura Finetek, Torrance, CA) for frozen sectioning or processed for paraffin sectioning. The cut planes were either sagittal at the midline or transverse at the midbody. For histological evaluation, dewaxed 5-μm sections were stained with hematoxylin and eosin. Immunostaining was performed as previously described with modifications (Torchia *et al.*, 2009). Briefly, paraffin-embedded tissue was dewaxed and antigen retrieval performed using a Tris EDTA solution, pH 10, containing 0.05% Tween20. Sections were then blocked and incubated with the following primary antibodies: Krt1, 5, 10, 14, loricrin and filaggrin (Roop *et al.*, 1984; Yuspa *et al.*, 1989), Col IV (Abcam, Cambridge, MA), Itα6 (EMD Millipore, Billerica, MA), γ-tubulin (Abcam), α-tubulin (Sigma), phospho-histone H3 (Ser 10) (Cell Signaling, Danvers, MA), and Active Capase 3 (Cell Signaling). Appropriate secondary antibodies labeled with Alexa488 or 594 were applied and sections mounted on Vectashield Mounting Medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Staining detection was performed on a Nikon 90i microscope (Nikon, Melville, NY) using standard techniques. For BrdU *in vivo* labeling experiments, mice were injected with BrdU (Sigma) as previously described (Torchia *et al.*, 2007). Tissue was then harvested after 2 hours, fixed, embedded in paraffin, and stained as described above using rat anti-BrdU antibody (Abcam). Staining for β-galactosidase (X-gal) activity was performed on 10-μm Optimal Cutting Temperature compound (Sakura Finetek) sections as previously described (Torchia *et al.*, 2007).

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization detection and enumeration of mouse centromeres was conducted at the University of Colorado Cancer Center Cytogenetic Core. Briefly, dewaxed sections were hybridized with SpectrumRed-labeled Poseidon. All Mouse Centromere Probe (KI-30500, Kreatech Diagnostics, Durham, NC). Chromatin was counterstained with DAPI (Vectashield Mounting Medium, Vector Laboratories). Analysis was performed using an epifluorescence microscope using single interference filter sets for red (Texas red), blue (DAPI), and triple (blue, red, green) band-pass filters. The number of signals was scored in at least 30 nuclei per selected area in each specimen. Images were acquired and merged using CytoVision application (Leica Microsystems, Buffalo Grove, IL).

Cell counting and statistical analyses

To enumerate the number of % keratinocytes that were positive for BrdU or phospho-histone H3, three separate embryos of each genotype were analyzed. Tissue sections from dorsal and ventral skins at E13.5 were stained for either BrdU incorporation/Krt14 or phospho-histone H3/Krt14. The number of BrdU- or phospho-histone H3–positive cells were scored at ×200 magnification after counting on average 294 and 575 Krt14–positive keratinocytes/embryo, respectively. Similar analysis was performed for active caspase 3–/Krt14–stained tissue sections with an average of 587 Krt14–positive cells/embryo counted. Dorsal skin was analyzed in two separate embryos for each embryonic time point. Statistical tests shown in Figure 5c (phosho-histone H3) were performed using GraphPad Prism v5 (Graph Pad Software, La Jolla, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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